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A novel class of small amphipathic peptides affect aerial hyphal growth and surface hydrophobicity in *Ustilago maydis*

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***Ustilago maydis*, a fungal pathogen of corn, can alternate between yeast-like and filamentous growth. This dimorphic switch is governed by the mating-type loci. We have identified an abundant class of small SDS-insoluble cell wall proteins, designated repellents, specifically present in the filamentous form. Genetic analysis revealed that these peptides are processed from a single precursor protein, Rep1. Rep1 comprises 652 amino acids with a leader sequence for secretion. A characteristic feature of Rep1 is 12 repeats of a 37 amino acid consensus sequence; 10 of these repeats are separated by Kex2 protease cleavage sites. In *Δrep1* mutants formation of aerial hyphae and surface hydrophobicity were reduced dramatically. This and the fact that expression of *rep1* is regulated by the mating-type loci indicates that repellents play a structural role in the formation of aerial hyphae.**

Keywords: aerial hyphae/hydrophobin/KEX2 protease/protein secretion/*Ustilago maydis*

Introduction

The heterobasidiomycete *Ustilago maydis* is the causal agent of corn smut disease and is characterized by its dimorphic mode of growth. Haploid sporidia grow yeast-like and are non-pathogenic; fusion of haploid cells results in a dikaryon that grows filamentous. This form needs the plant to differentiate and complete its life cycle with karyogamy and production of diploid teliospores (see Christensen, 1963; Banuett and Herskowitz, 1988; Banuett, 1992). Formation of the filamentous dikaryon can be monitored on complete medium containing charcoal where the aerial hyphae cause a fuzzy appearance (Fuz^+ phenotype) of colonies (Day and Anagnostakis, 1971; Banuett and Herskowitz, 1988).

In *U.maydis*, cell fusion, the morphological switch and subsequent pathogenic development are controlled by the *a* and *b* mating-type loci. Two haploid cells can fuse and form an infectious dikaryon only when they carry different alleles in both *a* and *b* (Rowell and DeVay, 1954; Rowell, 1955; Holliday, 1961). Such combinations are designated compatible.The *a* locus, with two alleles *a1* and *a2* (Rowell and DeVay, 1954; Rowell, 1955; Holliday, 1961; Puhalla, 1968), controls cell fusion through a pheromone-based recognition system (Bölker *et al.*, 1992; Spellig *et al.*, 1994). After cell fusion, the *a* locus is needed for maintenance of hyphal growth and this results from autocrine activation of the pheromone response pathway (Banuett and Herskowitz, 1989; Bölker *et al.*, 1992; Spellig *et al.*, 1994). The multiallelic *b* locus (Rowell and DeVay, 1954; Puhalla, 1968) controls all post-fusion steps of pathogenic development. *b* encodes a pair of unrelated homeodomain proteins, bE and bW (Schulz *et al.*, 1990; Gillissen *et al.*, 1992). These proteins dimerize only when they are derived from different alleles (Kämper *et al.*, 1995) and are then able to trigger pathogenic development.

Here we describe an unusual novel gene, *rep1*, that encodes the precursor for a class of short amphipathic peptides. These peptides appear to cause surface hydrophobicity and have a structural role in development of aerial hyphae.

Results

Identification of an abundant filament-specific cell wall protein

Protein patterns of isolated cell walls of the diploid strain FBD11 (*a1 a2 b1 b2*) displaying filamentous growth on charcoal plates and the nearly isogenic strain FBD11-21 (*a1 a2 b2 b2*) growing yeast-like on the same medium were compared. To this end, cell walls were treated with hot SDS followed by an extraction with trifluoroacetic acid (TFA). SDS–PAGE of the hot SDS extracts showed a very complex pattern of polypeptides, and no major differences were observed between both strains (not shown). Among the proteins which were insoluble in SDS but soluble in TFA, an abundant polypeptide was visible in the cell wall preparation of FBD11 but absent from FBD11-21 (Figure 1). This polypeptide, Rep1-2, had an apparent mol. wt of ~8 kDa. Western analysis using antibodies raised against Rep1-2 confirmed that Rep1-2 is present neither in the SDS-soluble cell wall fraction of FBD11 nor in the SDS-soluble and insoluble cell wall fractions of FBD11-21 (not shown). Isolated Rep1-2 was soluble in SDS (2%), TFA and formic acid (50–100%), but was largely insoluble in water, aqueous ethanol (0–80%), 10% TFA, 10% formic acid or 10% isopropanol (not shown). Rep1-2 could be released from SDS-treated cell wall preparations with TFA but not with 5 M NaCl. This indicates that, under physiological conditions, Rep1-2 is a filament-specific insoluble cell wall protein.

Cloning and characterization of the rep1 gene

The N-terminal sequence of Rep1-2 was determined to be TDYSACKKYVSSYNAGYNVYSINENKLIDLSDATVK.

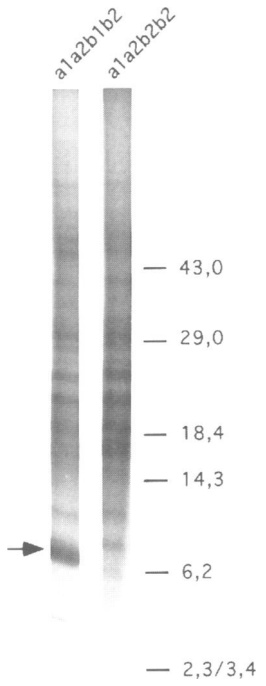

Fig. 1. Differential expression of Rep1-2. Protein extracts of the filamentous growing strain FBD11 (*a1 a2 b1 b2*) and the yeast-like growing strain FBD11-21 (*a1 a2 b2 b2*) were prepared as TFA extracts of SDS-treated cell walls and analysed by SDS–PAGE. The arrow indicates Rep1-2 that is only present in cell walls of FBD11. Molecular weight markers are given in kDa. Note that the 2.3 and 3.4 kDa markers do not separate under these conditions.

With the help of degenerate oligonucleotide primers representing amino acid residues 1–7 and 23–29 (see Materials and methods), a fragment of 99 bp could be amplified by PCR from a cDNA library derived from FBD11. This fragment was used to isolate genomic and cDNA clones. About 50 out of 2000 cDNA clones hybridized with the 99 bp fragment, indicating that the respective gene, *rep1*, is highly expressed. Sequencing of six independent cDNA clones differing in length revealed that they were all derived from the same gene. To the genomic and cDNA sequences an open reading frame (ORF) for a polypeptide of 652 amino acids with a predicted mol. wt of 71 330 Da could be assigned (Figure 2). No indications were found for the presence of introns. The coding sequence of *rep1* is preceded by a stretch of at least 138 nucleotides that are not translated. The sequence surrounding the start codon is similar to the fungal consensus CA(c/a)(a/c) ATGNC (Ballance, 1990). A second putative translation initiation site at position +51 is not in a favourable sequence context (Figure 2).

The deduced amino acid sequence of Rep1 (Figure 2) shows the following salient features: at the N-terminus, we find a typical signal sequence for secretion, followed by 12 repeats varying in length between 37 and 55 amino acids. Comparison of these repeats reveals a highly conserved 37 amino acid consensus sequence that displays an amphipathic character (Figure 3). A hydropathy plot of Rep1 shows a regular alternating pattern of hydrophilic and hydrophobic regions, interrupted by a hydrophilic proline-rich region between repeats 10 and 11 (Figure 3B). The amino acid sequence determined for the isolated peptide Rep1-2 starts at the beginning of the second amphipathic repeat of Rep1 (Figures 2 and 3A), indicating that Rep1-2 is derived from Rep1 by processing events. The N-terminal threonine of Rep1-2 is preceded by the motif Lys–Arg, which represents a typical Kex2 processing site (Fuller *et al.*, 1988). The yeast *KEX2* gene product cleaves the α-factor precursor at the carboxyl side of Lys–Arg sites and has become the paradigm for this class of processing enzymes. The C-terminal basic residues are then removed by the action of a B-type carboxypeptidase, the product of the *KEX1* gene (Fuller *et al.*, 1988). A Kex2-like activity has been demonstrated previously to reside in the endoplasmatic reticulum of *U.maydis* (Park *et al.*, 1994). Since the N-terminal 10 repeats each contain a Kex2 processing site, cleavage of Rep1 by the concerted action of a Kex2-like and a Kex1-like protease would generate not only Rep1-2 but also nine additional small polypeptides (designated Rep1-1–Rep1-10), ranging in size from 35 to 53 amino acid residues, and one larger polypeptide (Rep1-C) of 228 amino acid residues (see Figure 2 and Table I).

Identification of additional Rep1 processing products in cell walls of filaments

The Rep1-2 peptide originally isolated from cell walls yielded an unambiguous amino acid sequence. Therefore, the question arose as to why the other predicted processing products of Rep1 were not found in this preparation. Since Rep1-2 was soluble in SDS only after TFA extraction, we surmised that the other peptides might be insoluble in SDS even after extraction with TFA. Therefore, the extraction procedure was modified such that after TFA treatment proteins were solubilized in 50% formic acid instead of 2% SDS. Proteins contained in this fraction were separated by HPLC (see Materials and methods). From the complex pattern of peaks obtained (Figure 4), major peak fractions were analysed both by N-terminal sequencing and mass spectroscopy. The results are summarized in Table I. Except for peptides Rep1-3, Rep1-6 and Rep1-C, all predicted processing products could be identified. From their N-terminal sequence and their molecular mass, the peptides representing repeats 1, 5, 7, 8, 9 and 10 corresponded exactly to the expected products produced by Kex2–Kex1 cleavage. For peptides Rep1-5 and Rep1-8 we observe removal of the lysine residue preceding the Lys–Arg sequence which is consistent with the properties of the Kex1 carboxypeptidase that specifically removes basic residues from the C-terminus. Peptides Rep1-2 and Rep1-4 were identified only on the basis of their N-terminal sequence. Peptide 1-4 lacks a lysine residue expected at the N-terminus, which indicates an additional processing event. For these peptides, masses could not be determined, which might be explained by heterogeneous C-terminal processing sites. In those cases where masses of peptides could be determined, no evidence for post-translational modifications was found. These data show that Rep1 is processed and that most products, if not all, are contained in cell walls of filaments.

rep1 expression is regulated by the mating-type loci

Since Rep1 processing products were detected only in the filamentous stage, we have investigated whether *rep1*

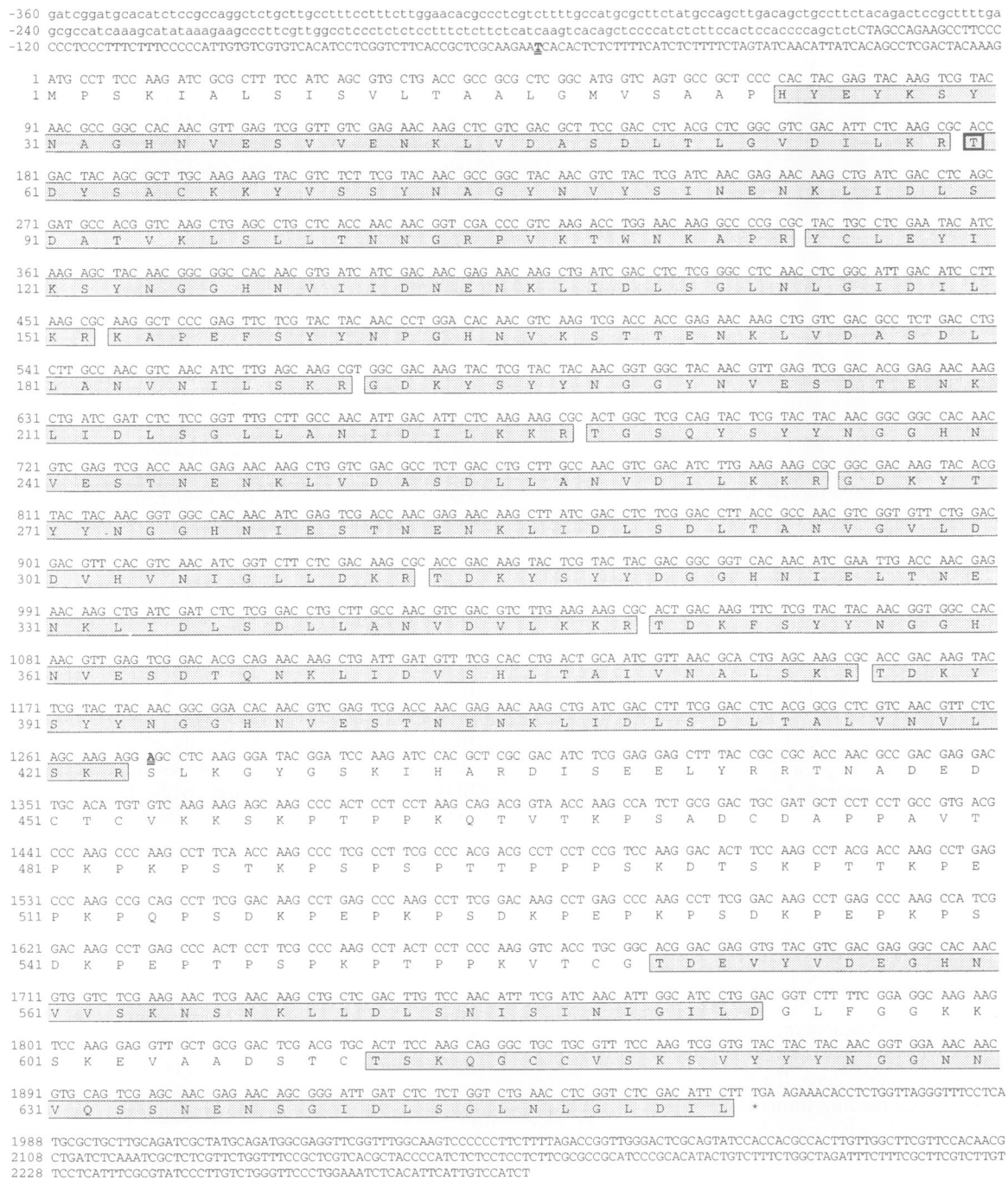

Fig. 2. Nucleotide sequence and deduced amino acid sequence of *rep1*. The ORF extends from nucleotide +1 to nucleotide +1956 (GenBank accession No. U56826). The nucleotide sequence of the cDNA is given in upper case. Polyadenylation was observed at the most 3′ C or T residue of the sequence shown. Double underlined nucleotides at positions –59 and 1271 written in bold type indicate the extent of the deletion in pΔrep1. The Rep1 protein is characterized by the presence of 12 repeats indicated by shaded boxes. The boxed threonine residue indicates the N-terminus of Rep1-2.

expression is regulated by the mating-type loci. Levels of *rep1* mRNA were low in the haploid strains FB1 (*a1 b1*) and FB2 (*a2 b2*) which had been grown for 48 h on solid CM charcoal medium (Figure 5). When these compatible haploid strains were crossed, the filamentous dikaryon formed and the level of *rep1* mRNA increased ~50-fold. In the diploid strain FBD11 that is heterozygous for *a* and *b* and grows filamentous, *rep1* expression levels were even higher than in the cross. We presume that this is due to the fact that the diploid strain FBD11 can switch to filamentous growth more efficiently than a mixture of haploid strains that have to fuse first. Expression of *rep1* in FBD11 increased significantly after 24 h and reached a maximum after 48 h, which correlated with the appearance of filaments. In diploid strains which grow yeast-like because they are homozygous in either *a* or *b*, *rep1* was found to be expressed at low levels (Figure 5). These data show that expression of *rep1* is regulated by the mating-type loci.

Disruption of the rep1 gene affects aerial growth of filaments and surface hydrophobicity

The *rep1* gene was inactivated in FB1 and FB2 by gene replacement with the disruption construct pΔrep1. In this plasmid, the region from nucleotide –59 to +1271 of *rep1* was replaced by a hygromycin resistance cassette (see Materials and methods and Figure 2). This deletion removes all but the last two repeats. Since these repeats lack both a signal sequence and KEX2 protease cleavage sites, it is very likely that this deletion leads to complete loss of function. In both strains, 25% of the hygromycin-resistant transformants showed a targeted inactivation of *rep1*, indicating that *rep1* is non-essential. Growth rates of wild-type and mutant strains were comparable in different media and, in compatible combinations, Δ*rep1* mutant strains were neither affected in pathogenicity nor in formation and viability of teliospores (not shown).

When the wild-type strains FB1 and FB2 were crossed on solid charcoal medium aerial filaments formed (Fuz$^+$) (Figure 6A) while, in a cross of compatible Δ*rep1* mutants, formation of these aerial filaments was reduced dramatically (Figure 6A). However, at the edge of these colonies, filaments that were in contact with the agar surface were observed for both the wild-type and mutant strains (Figure 7A and B).

To analyse this phenomenon in more detail, compatible

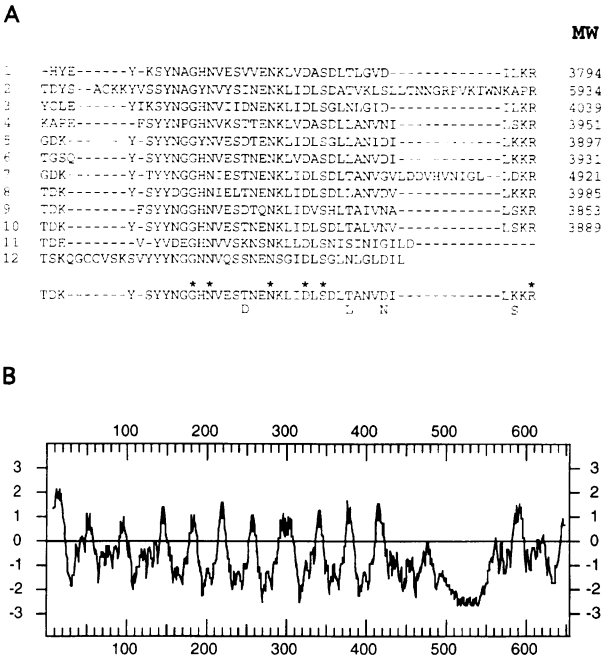

Fig. 3. Structural features of Rep1. (**A**) Sequence alignment of repeated amino acid motifs in Rep1. The 37 amino acid consensus sequence is given below with invariant amino acids indicated by astersiks. All repeats except numbers 2, 11 and 12 end with a KR sequence, which is a typical Kex2-like cleavage site (Fuller *et al.*, 1989). Repeat number 2 ends with the dipeptide PR that is also known to be processed by Kex2 in *U.maydis* (Park *et al.*, 1994). Kex2 has an endopeptidase activity; the basic amino acids of the recognition site are removed subsequently by Kex1 (Fuller *et al.*, 1989). The molecular weights of predicted processing products are indicated. (**B**) Hydropathy pattern of Rep1 produced by the program DNA Strider 1.0 (Marck, 1988) using the parameters of Kyte and Doolittle (1982).

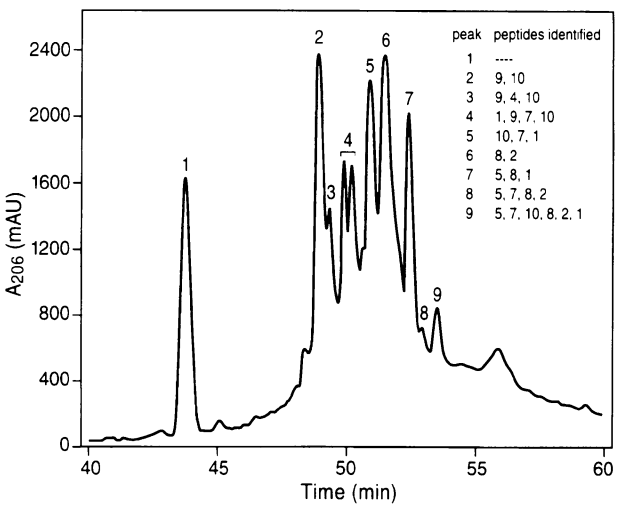

Fig. 4. Cell wall fractions contain several processing products of Rep1. SDS-treated walls of FBD11 were extracted with TFA. After removal of the acid, proteins were solubilized in 50% formic acid and subjected to HPLC. The elution profile is shown. Peaks are numbered, and proteins identified in individual peak fractions by mass spectroscopy and N-terminal sequencing are indicated on the right (see Table I). Partial formylation of peptides due to the presence of formic acid during the extraction can explain the occurrence of specific peptides in more than one peak.

Table I. Predicted and experimentally identified peptides derived from Rep1

Peptide	N-terminal aa sequence expected	N-terminal aa sequence determined	Mass expected[a]	Mass determined from aa sequence
Rep1-1	HYEYK	HYEYK	3794.2	3794.8
Rep1-2	TDYSA	TDYSA	5933.7	n.d.[b]
Rep1-3	YCLEY	n.d.	4038.6	n.d.
Rep1-4	KAPEF	APEF[c]	3951.4	n.d.
Rep1-5	GDKYS	GDKYS	3769.1	3767.8
Rep1-6	TGSQY	n.d.	3931.2	n.d.
Rep1-7	GDKYT	GDKYT	4921.3	4920.0
Rep1-8	TDKYS	TDKYS	3857.2	3857.6
Rep1-9	TDKFS	TDKFS	3853.2	3851.6
Rep1-10	TDKYS	TDKYS	3889.2	3888.6
Rep1-C[d]	SLKGY	n.d.	24346.4	n.d.

[a]Averaged expected masses were calculated considering removal of the C-terminal basic amino acids.
[b]n.d. means that the respective peptide was not found or its mass could not be determined.
[c]Sequencing indicates removal of K residue from N-terminus. This would yield a product of the expected mass 3823.2 Da.
[d]Rep1-C is the predicted C-terminal peptide remaining after processing of Rep1; it contains two repeat sequences not flanked by processing sites.

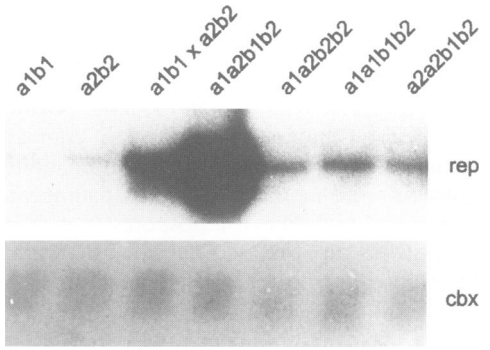

Fig. 5. *rep1* mRNA levels in strains differing at the mating type loci. Total RNA was prepared from cultures grown for 48 h on solid CM medium containing charcoal and analysed by Northern blotting. A 99 bp PCR fragment encoding amino acids 1–29 of *rep1* was used as hybridization probe (upper panel). Overexposure of the film was intentional to visualize the weak signals in haploid strains. Loading of equal amounts of RNA was confirmed by hybridization of the filter with a probe derived from the gene conferring carboxin resistance (lower panel). The genotype of strains is indicated: FB1 is *a1 b1*, FB2 is *a2 b2*, FBD11 is *a1 a2 b1 b2*, FBD11-21 is *a1 a2 b2 b2*, FBD11-7 is *a1 a1 b1 b2* and FBD12-17 is *a2 a2 b1 b2*.

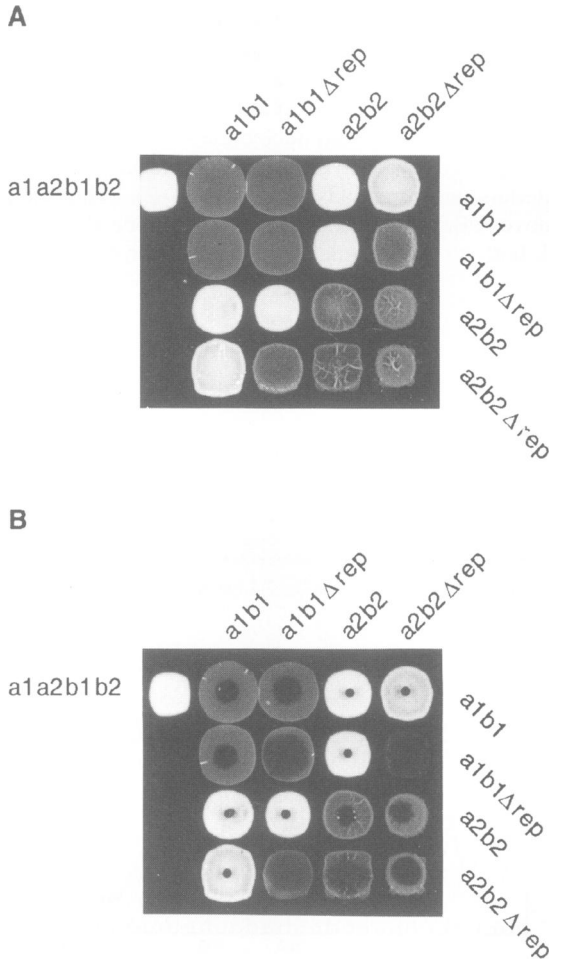

Fig. 6. The phenotype of Δ*rep1* mutants in crosses. (**A**) Strains indicated at the top and on the right were spotted alone or in combination on CM charcoal plates. Strains used are FB1 (*a1 b1*), FB1Δrep1, FB2 (*a2 b2*), FB2Δrep1. The white fuzzy appearance of the colonies indicates normal development of dikaryotic hyphae, the smooth appearance indicates that hyphal development does not occur or is strongly impaired. (**B**) The same plate as in (A) but water droplets (visible as dark spots) were placed on top of the colonies to measure surface hydrophobicity. Spreading of water droplets is inversely correlated with hydrophobicity. On the far left in (A) and in (B), FBD11 (*a1 a2 b1 b2*) was spotted alone and without a water droplet as a reference for the Fuz^+ phenotype.

wild-type cells or Δ*rep1* mutant cells in water were mixed and placed in small droplets on the hydrophobic surface of silanized glass slides. After 24 h of incubation, the average number and length of dikaryotic filaments formed in the aqueous phase was indistinguishable in both crosses (Figure 7C and D). However, in the cross of wild-type cells, very long hyphae (≥350 μm) could be observed that had escaped from the water droplet and had grown onto the dry hydrophobic surface (Figure 7E). Such hyphae were absent in the cross of the Δ*rep1* strains (Figure 7F). This could indicate that mutants in *rep1* are affected in surface hydrophobicity. To test for this directly, water droplets were spotted onto colonies of haploid wild-type and Δ*rep1* strains and various strain combinations. Droplets spread moderately on the surface of the haploid wild-type strains (Figure 6B). In contrast, water droplets did not spread on the hydrophobic surface of aerial hyphae formed in the cross of wild-type cells. On the surface of haploid Δ*rep1* strains and on colonies where the compatible Δ*rep1* strains had been mixed, water droplets spread completely (Figure 6B). These data show that the peptides derived from *rep1* act as repellents and are required for surface hydrophobicity and aerial growth.

Discussion

The *rep1* gene of *U.maydis* is expressed abundantly in the filamentous stage where it is required for the development of aerial hyphae. *rep1* encodes a protein of 652 amino acids which is processed into small peptides related in sequence. These peptides, designated repellents, are secreted and are found in the cell wall where they mediate surface hydrophobicity.

Although deletion of *rep1* did not affect viability of the haploid strains, we observed two clear phenotypes in Δ*rep1* mutants. First, mutant strains showed a lower surface hydrophobicity compared with wild-type strains. Second, when two compatible Δ*rep1* strains were crossed, formation of aerial hyphae was strongly reduced as compared with a cross of compatible wild-type cells. In an aqueous environment, however, Δ*rep1* mutants are able to form filaments, indicating that cell fusion and filamentous growth *per se* are not affected by the mutation. Apparently, the loss of surface hydrophobicity in the Δ*rep1* strains specifically prevents formation of aerial hyphae. Surface hydrophobicity may promote escape of hyphae from the aqueous environment and could protect aerial hyphae against desiccation.

The repellents described here resemble in function, location and solubility the hydrophobins that represent a large family of hydrophobic proteins found in diverse groups of fungi (see Wessels, 1994). Despite these common properties, repellents and hydrophobins display no similarities at the amino acid level. Hydrophobins are ~100 amino acids in length and are characterized by eight conserved cysteine residues and a typical hydropathy pattern (Wessels, 1994). Most of the 35–53 amino acid long repellents do not contain cysteine residues and

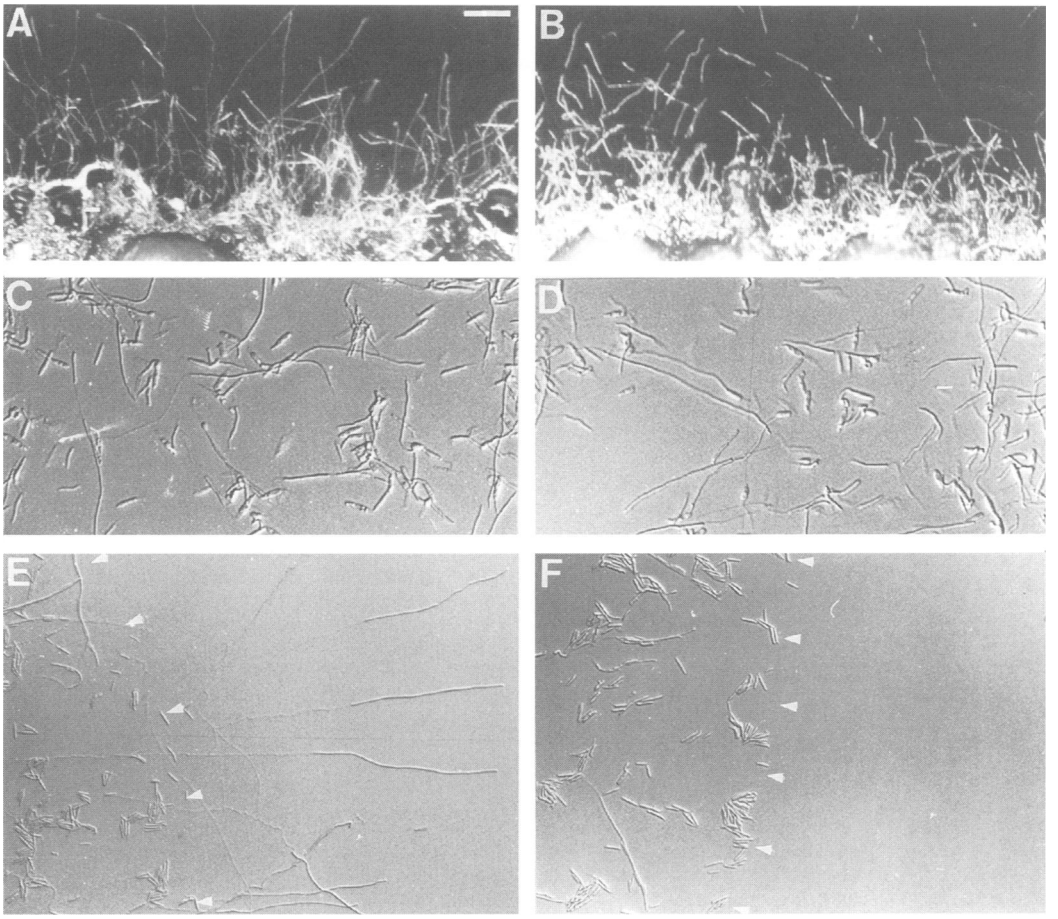

Fig. 7. Microscopic analysis of filament formation in Δ*rep1* mutants. The left panel (**A**, **C** and **E**) represents a cross between FB1 and FB2, the right panel (**B**, **D** and **F**) represents a cross between FB1Δrep1 and FB2Δrep1. The first row (A and B) is a view of the edge of the colonies growing on charcoal plates showing normal hyphal development on the agar surface. The second row (C and D) shows filament formation in water droplets after 24 h that occurs to a similar degree for both strain combinations. In the last row (E and F), filament escape from water droplets is shown. The edge of water droplets is indicated by white arrowheads. Only filaments formed in the cross of wild-type strains (E) can leave the aqueous environment. Bar shown in (A) represents 60 μm in (A) and (B) and 40 μm in (C), (D), (E) and (F), respectively.

their hydropathy pattern bears no resemblance to that of hydrophobins. The hydrophobins are structural proteins involved in formation of aerial hyphae and fruiting bodies (Wessels *et al.*, 1991a,b). In addition, hydrophobins have been implicated in attachment of hyphae to hydrophobic surfaces, a function that might also be important for plant–pathogen interactions (Wösten *et al.*, 1994b; see Templeton *et al.*, 1994). The most extensively characterized hydrophobin Sc3 of *Schizophyllum commune* is secreted in monomeric form when the fungus grows in submerged culture. During formation of aerial hyphae or growth on a hydrophobic surface, Sc3 assembles at the interface between the hydrophilic cell wall and the hydrophobic environment to form a hydrophobic layer (Wessels *et al.*, 1991a,b; Wösten *et al.*, 1993, 1994a,b). In mutants of *S.commune* lacking the hydrophobin Sc3, formation of aerial hyphae as well as attachment of hyphae to hydrophobic surfaces was affected, but hyphal growth in aqueous medium was indistinguishable from wild-type strains (Wösten *et al.*, 1994b; van Wetter *et al.*, 1996). This parallels what is observed for the Δ*rep1*mutants of *U.maydis* which can form hyphae only in an aqueous environment, and raises the possibility that, in *U.maydis*, repellents may have replaced hydrophobins in function. The alternative possibility that repellents might function indirectly, e.g. by serving as anchors for hydrophobins, appears unlikely since we were unable to detect abundant hydrophobins in filaments. The view that repellents may be able to replace hydrophobins is supported further by the high expression level of *rep1* (~2.5% of total mRNA), which is in the same range as found for hydrophobins. The amphipathic profile of the repellents might suggest that they could self-assemble at the surface of the cell wall in a way similar to hydrophobins. Their hydrophobic parts would be oriented towards the air, thus creating a hydrophobic coating. Since repellents could not be extracted from the cell wall by treatment with 5 M NaCl, non-ionic forces must play a major role in this interaction. For hydrophobins, it was suggested initially that aggregation involves intermolecular disulfide bridges. More recent experiments do not support this possibility but also point to the involvement of non-ionic forces governing self-assembly (de Vries *et al.*, 1993).

For repellents and hydrophobins alike, it should be fascinating to determine how these insoluble protein layers are formed from proteins so different in primary structure. It is also tempting to speculate that repellents might be more widespread in the fungal kingdom. In the past, these proteins might have escaped detection because of their unusual properties.

Materials and methods

Strains and plasmids

Cloning in *Escherichia coli* was done in DH5α (BRL). The haploid *U.maydis* strains FB1 (*a1 b1*) and FB2 (*a2 b2*) (Banuett and Herskowitz, 1989) are non-pathogenic (Tum$^-$) and grow yeast-like (Fuz$^-$) on complete solid medium containing charcoal. The stable diploid strains FBD11 (*a1 a2 b1 b2*, Tum$^+$, Fuz$^+$), FBD11-7 (*a1 a1 b1 b2*, Tum$^+$, Fuz$^-$), FBD12-17 (*a2 a2 b1 b2*, Tum$^+$, Fuz$^-$) and FBD11-21 (*a1 a2 b2 b2*, Tum$^-$, Fuz$^-$) have been described (Banuett and Herskowitz, 1989). For cloning in *E.coli*, the plasmids pUC19 and pBluescript II SK (Stratagene) were used. For transformation of *U.maydis*, a hygromycin resistance cassette was isolated from the *U.maydis* vector pCM54 (Tsukuda *et al.*, 1988). A cDNA library of *U.maydis* FBD11 was prepared from RNA of a culture (Fuz$^+$) grown on CM charcoal for 48 h at 22°C. The cDNA was cloned as *Eco*RI fragments in λgt10 (Amersham) (Schauwecker *et al.*, 1995). A cosmid library of FBD11 was constructed by cloning partial *Mbo*I fragments into the *Bam*HI site of pUMcos (Bölker *et al.*, 1995).

Growth conditions and media

U.maydis was grown at 28°C in YEPS (Tsukuda *et al.*, 1988) or CM medium (Holliday, 1974). To allow for filamentous growth, the fungus was grown at 22°C on CM plates containing 1% charcoal (Holliday, 1974). Maize plants of the varieties Early Golden Bantam and Gaspar Flint were infected as described (Schauwecker *et al.*, 1995).

Molecular techniques

Standard molecular techniques followed Sambrook *et al.* (1989). *U.maydis* chromosomal DNA was isolated as described by Hoffman and Winston (1987). RNA was isolated according to Schauwecker *et al.* (1995). DNA and RNA were blotted on a Nylon filter (Hybond N$^+$, Amersham) and were hybridized under conditions described by Church and Gilbert (1984) and Timberlake (1986), respectively. For radioactive labelling of DNA, the Megaprime DNA Labelling System from Amersham was used. Nucleotide sequences were determined with alkaline denatured plasmid DNA as described by Sanger *et al.* (1977) using the T7 DNA polymerase (T7 Sequencing Kit, Pharmacia).

Isolation of rep1 and a corresponding cDNA

Degenerate oligonucleotide primers representing amino acids 1–7 (CTC-TAGAGCTCACNGAYTAYWSNGCNTGYAAR) and 23–29 (GACG-TCTCGAGCTRTADRANTTYTTRCTYTTR) of Rep1-2 containing linkers for *Sac*I and *Xho*I, respectively, were used in PCR with DNA from the cDNA library of FBD11 as template. A 99 bp fragment encoding amino acids 1–29 of Rep1-2 was isolated and cloned in the *Sac*I and *Xho*I sites of pBluescript. This fragment was used to isolate a genomic clone, pMN4.2, encompassing a 4.2 kb *Mlu*I–*Nde*I fragment, and a full-length cDNA clone, pRep1c, from the cosmid and cDNA libraries, respectively.

Deletion of rep1 in U.maydis

The deletion construct pΔrep1 was made by replacing a 1.3 kb fragment from the coding region of *rep1* (see Figure 2) by a hygromycin resistance cassette. To generate the *rep1* deletion, oligonucleotides with *Not*I linkers at their 5′ ends (ATTTGCGGCCGCTTCTTGCAAGCGCTGTAGTCGG and ATTTGCGGCCGCGCCTCAAGGGATACGGATCC) were used in PCR reactions with DNA polymerase of *Thermus flavus* (Biozym) and with pMN4.2 as template. After digestion with *Not*I, the amplified fragment was ligated to yield pMN3.1. To extend the deletion, a 248 bp *Tfi*I–*Eco*47III fragment (position –59 to 189, Figure 2) was removed from pMN3.1 and the plasmid backbone ligated after filling in the sticky end at the *Tfi*I site, resulting in pMN2.3. The hygromycin B resistance gene (*hyg*B) under control of the *hsp*70 promotor was excised as a *Pvu*II fragment from pCM54, ligated to *Not*I linkers and cloned into the *Not*I site of pMN2.3, to yield pΔrep1.

For gene replacement, *U.maydis* was transformed with pΔrep1 linearized with *Sph*I as described by Schulz *et al.* (1990). Gene replacement was verified by Southern analysis.

Preparation of cell walls and protein extracts

Fungal cells were broken by grinding in liquid nitrogen. Hot 2% SDS-extracted walls were prepared as described (Wessels *et al.* 1991a,b) and subsequently extracted with TFA (Wösten *et al.*, 1993). After removal of the acid by a stream of nitrogen, extracts were taken up in SDS sample buffer (2% SDS, 20% glycerol, 0.02% bromophenolblue, 0.1 M Tris–HCl pH 6.8) and subjected to SDS–PAGE. If necessary, adjustments of pH were done by addition of 1 M Tris. In some experiments, TFA-extracted proteins were taken up in 50% formic acid (v/v).

Electrophoresis

SDS–PAGE was done in 10% SDS–polyacrylamide gels (0.31% bisacrylamide) with tricine as a buffering component (Schägger and von Jagow, 1987). After separation, proteins were stained with Coomassie Brilliant Blue G-250 or blotted onto a polyvinylidenedifluoride (PVDF) membrane (Milipore). The low molecular weight marker of Gibco BRL was used.

Identification of peptides encoded by rep1

Peptides encoded by *rep1* were purified by SDS–PAGE or by HPLC and then characterized by N-terminal sequencing or mass spectroscopy. Rep1-2 was identified initially by SDS–PAGE. The peptide was blotted onto a PVDF membrane, stained with Coomassie Brilliant Blue G-250 and a slice of the membrane containing the protein was cut out (Sambrook *et al.*, 1989). After destaining, the N-terminal sequence of Rep1-2 was determined.

Eight peptides encoded by *rep1* were identified after taking up TFA-soluble proteins of SDS-extracted cell walls of FBD11 in 50% formic acid. Peptides soluble in the aqueous formic acid were separated on a lichrosphere RP-18 (Merck, Darmstadt, Germany) column (2.1 mm× 10 cm) using a gradient of 0–80% acetonitrile in 0.1% TFA (0–60% in 60 min, 60–80% in 5 min). Peptides were detected by absorption at 206 nm. Individual peak fractions were subjected to N-terminal sequencing and mass spectroscopy analysis.

N-terminal sequencing of proteins was done on a 472A pulsed liquid phase sequencer (Applied Biosystems) and mass spectroscopy was performed on a API III Biomolecular Mass Analyzer (Perkin Elmer Sciex) according to the instructions of the manufacturers.

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